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PROTON TRANSPORTERS AND USES IN PLANTS

RELATED APPLICATION(S)

This application is a continuation of U.S. Application No. 09/644,039, entitled "Proton Transporters and Uses in Plants" by Roberto A. Gaxiola, filed August 22, 2000, which claims the benefit of U.S. Application No. 60/164,808, entitled "Proton Transporters and Uses in Plants" by Roberto Gaxiola, filed November 10, 1999. The entire teachings of the above applications are incorporated herein by reference.

GOVERNMENT SUPPORT

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BACKGROUND OF THE INVENTION

The prospects for feeding humanity as we enter the new millennium are formidable. The progressive salinization of irrigated land compromises the future of agriculture in the most productive areas of our planet (Serrano, R., *et al., Crit. Rev. Plant Sci., 13*:121-138 (1994)). Arid regions offer optimal photoperiod and temperature conditions for the growth of most crops, but suboptimal rainfall. Artificial irrigation has

solved the problem in the short term. However, water supplies always contain some dissolved salt, which upon evaporation gradually accumulates on the soils.

To grow in saline environments, plants must maintain a much lower ratio of Na^+/K^+ in their cytoplasm than that present in the soil. A need exists for crops having increased tolerance to salt.

SUMMARY OF THE INVENTION

The present invention relates to a transgenic plant which is tolerant to a salt, comprising one or more plant cells transformed with exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant. In one embodiment, the exogenous nucleic acid encodes AVP1 or a homologue thereof. In another embodiment, the present invention relates to a transgenic plant which grows in a concentration of a salt that inhibits growth of a corresponding non-transgenic plant. In a particular embodiment, the transgenic plant is tolerant to sodium chloride (NaCl) and the NaCl concentration is from about 0.2M to about 0.3M. In a another embodiment, the present invention relates to a transgenic plant which is tolerant to salt comprising (containing within some or all of its cells) an exogenous nucleic acid construct which is designed to overexpress AVP1 or designed to down regulate endogenous pyrophosphatase. In yet another embodiment, the invention relates to a transgenic plant obtained by introducing into a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant.

Also encompassed by the present invention are transgenic progeny and seeds of the transgenic plants described herein. Progeny transgenic plants grown from seed of transgenic plants are also described.

The present invention also relates to a construct comprising an AVP1 gene operably linked to a chimeric promoter designed to overexpress AVP1 or designed to down regulate endogenous pyrophosphatase. In one embodiment, the AVP1 gene is operably linked to a double tandem enhancer of a 35S promoter.

Plant cells (*e.g.*, root cells, stem cell, leaf cells) comprising exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant cell are also the subject of the present invention.

Also encompassed by the present invention is a method of making a transgenic plant which is tolerant to salt comprising introducing into one or more cells of a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells in the plant, thereby producing a transgenic plant which is tolerant to salt. For example, this can be carried out in a whole plant, seeds, leaves, roots or any other plant part. In one embodiment, the present invention relates to a method of making a transgenic plant which is tolerant to salt comprising introducing into one or more cells of a plant a nucleic acid construct which is designed to overexpress AVP1 to yield transformed cells thereby producing a transgenic plant which is tolerant to salt. The method can further comprise regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is tolerant to salt, thereby producing a transgenic plant which is tolerant to salt.

The present invention also relates to a method of making a transgenic plant which is larger than its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant which is larger than its corresponding wild type. The method can further comprise regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is tolerant to salt, thereby producing a transgenic plant which is larger than its corresponding wild type.

Transgenic plants produced by the methods of making a transgenic plant as described herein are also a subject of the present invention.

The present invention relates to a method of bioremediating soil comprising growing one or more transgenic plants and/or progeny thereof in the soil, wherein the transgenic plants and/or progeny thereof comprise exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant. The transgenic plants grow in the

soil, and in the process, take up cations from the soil. In one embodiment, the present invention relates to a method of removing one or more cations from a medium which can support plant growth (*e.g.*, soil, water) comprising growing one or more transgenic plants and/or progeny thereof in the medium, wherein the transgenic plants and/or progeny thereof comprise exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant.

The present invention provides for a method of increasing the yield of a plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby increasing the yield of the plant. The method can further comprise regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is larger than its corresponding wild type plant, thereby producing a transgenic plant which is larger than its corresponding wild type plant.

Also encompassed by the present invention is a method of making a transgenic plant (*e.g.*, an ornamental plant) having increased flower size compared to its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant having increased flower size compared to its corresponding wild type plant.

The present invention also provides for a method of producing a transgenic plant which grows in salt water, such as water in which the salt concentration is equivalent to that of seawater (*e.g.*, about 0.2M to about 0.4M), comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant which grows in salt water. The method can further comprise regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is larger than its corresponding wild type plant, thereby producing a transgenic plant which can grow in salt water.

The transgenic plants of the present invention can also be used to produce double transgenic plants which are tolerant to salt (about 0.2M to about 0.4M salt concentration). In one embodiment, the present invention relates to a double transgenic plant which is tolerant to salt comprising one or more plant cells transformed with exogenous nucleic acid which alters expression of a vacuolar pyrophosphatase and an Na^+/H^+ antiporter in the plant. In one embodiment, the vacuolar pyrophosphatase is AVP1 or a homologue thereof and the Na^+/H^+ antiporter is AtNHX1 or a homologue thereof. The present invention further relates to a transgenic progeny of the double transgenic plant, as well as seeds produced by the transgenic plant and a progeny transgenic plant grown from the seed.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are bar graphs showing the intracellular Na^+ and K^+ contents of wild-type yeast strains and of yeast strains carrying various mutations affecting sodium tolerance; values are the mean of two determinations, and bars represent the standard deviations.

Figure 2 is alignment of the deduced amino acid sequences of NhX1 homologue from Arabidopsis *AtNHX1* (SEQ ID NO:1), human *HsNHE-6* (SEQ ID NO: 2) and yeast *ScNHX1* (SEQ ID NO:3); identical residues are in black boxes, and dashes indicate gaps in the sequence, * above alignment denote putative amiloride binding site from human *NHE1* (^{163}DVF-FLFLLPPI^{173}) (SEQ ID NO: 4).

Figure 3A is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast prevacuolar compartment; Nhx1 (Na^+/H^+ antiporter), Vmal (vacuolar membrane H^+-ATPAse), Gefl (yeast CLC chloride channel), Enal (plasma membrane Na^+-ATPase).

Figure 3B is a schematic representation of a working model of the transporters involved in sodium sequestration at the yeast prevacuolar compartment shown in Figure 3A, which also includes Avp1 (*A. thaliana* vacuolar pyrophosphate-energized proton pump).

DETAILED DESCRIPTION OF THE INVENTION

Producing salt-tolerant plants using genetic engineering requires the identification of the relevant genes. Physiological studies suggest that salt exclusion in the root and/or salt sequestration in the leaf cell vacuoles are critical determinants for salt tolerance (Kirsch, M., *et al., Plant Mol. Biol., 32*:543-547 (1996)). Toxic concentrations of sodium chloride (NaCl) build up first in the fully expanded leaves where NaCl is compartmentalized in the vacuoles. Only after their loading capacity is surpassed, do the cytosolic and apoplasmic concentrations reach toxic levels, ultimately leading to loss of turgor, *ergo* plant death. It has been suggested that hyperacidification of the vacuolar lumen via the V-ATPase provides the extra protons required for a Na^+/H^+ exchange-activity leading to the detoxification of the cytosol (Tsiantis, M.S., *et al., Plant J., 9*:729-736 (1996)). Salt stress increases both ATP- and pyrophosphate (PPi)-dependent H^+ transport in tonoplast vesicles from sunflower seedling roots. Salt treatments also induce an amiloride-sensitive Na^+/H^+ exchange activity (Ballesteros, E., *et al., Physiologia Plantarum, 99*:328-334 (1997)). In the halophyte *Mesembryanthemum crystallinum*, high NaCl stimulates the activities of both the vacuolar H^+-ATPase (V-ATPase) and a vacuolar Na^+/H^+ antiporter in leaf cells. As described herein, the plant components involved in the intracellular detoxification system have been identified by complementing salt-sensitive mutants of the budding yeast *Saccharomyces cerevisiae*. As also described herein, *Arabidopsis thaliana* has been used as a host model plant to demonstrate that overexpression of these genes results in salt tolerance in the plant.

Accordingly, the present invention is directed to transgenic plants which are tolerant to one or more salts. As used herein, the term "salt" refers to any salt, such as NaCl, KCl, and/or $CaCl_2$. In one embodiment, the transgenic plants of the present invention comprise one or more plant cells transformed with exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant. Any suitable vacuolar pyrophosphatase, several of which have been cloned, can be used in the compositions

and methods of the present invention (*e.g.*, Sarasian, Z., *et al., Proc. Natl. Acad. Sci., USA, 89:*1775-1779 (1992); Jenslerchl, *et al., Molec. Biol., 29:* 833-840 (1995); Kim, Y., *et al., Plant Physiol., 106:*375-382 (1994)). As used herein, nucleic acid which "alters expression of vacuolar pyrophosphatase" includes nucleic acid which enhances (promotes) or inhibits expression of vacuolar pyrophosphatase in the transgenic plant. In a particular embodiment, the present invention relates to a transgenic plant which is tolerant to salt comprising an exogenous nucleic acid construct which is designed to overexpress AVP1 (Sarasian, Z., *et al., Proc. Natl. Acad. Sci., USA, 89:*1775-1779 (1992)) or designed to downregulate endogenous vacuolar pyrophosphatase. The present invention also encompasses transgenic plants which grow in a concentration of salt that inhibits growth of a corresponding non-transgenic plant. Transgenic progeny of the transgenic plants, seeds produced by the transgenic plant and progeny transgenic plants grown from the transgenic seed are also the subject of the present invention. Also described herein are plant cells comprising exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant cell.

Any suitable nucleic acid molecule which alters expression of vacuolar pyrophosphatase in the plant can be used to transform the transgenic plants in accordance with the present invention. Exogenous nucleic acid is a nucleic acid from a source other than the plant cell into which it is introduced or into a plant or plant part from which the tansgenic part was produced. The exogenous nucleic acid used for transformation can be RNA or DNA, (*e.g.*, cDNA, genomic DNA). In addition, the exogenous nucleic acid can be circular or linear, double-stranded or single-stranded molecules. Single-stranded nucleic acid can be the sense strand or the anti-sense strand.

The exogenous nucleic acid can comprise nucleic acid that encodes a vacuolar pyrophosphatase protein (an exogenous vacuolar pyrophosphatase), such as AVP1, a functional portion thereof (peptide, polypeptide), or a homologue thereof, and/or nucleic acid that alters (enhances, inhibits) expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced. As used herein a "functional portion" of a nucleic acid that encodes a vacuolar

pyrophosphatase protein is a portion of the nucleic acid that encodes a protein or polypeptide which retains a function characteristic of a vacuolar pyrophosphatase protein. In a particular embodiment, the nucleic acid encodes AVP1, a functional portion or a homologue thereof.

Nucleic acid that alters (enhances, inhibits) expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced includes regulatory sequences (*e.g.,* inducible, constitutive) which function in plants and antisense nucleic acid. Examples of regulatory sequences include promoters, enhancers and/or suppressors of vacuolar pyrophosphatase. The nucleic acid can also include, for example, polyadenylation site, reporter gene and/or intron sequences and the like whose presence may not be necessary for function or expression of the nucleic acid but can provide improved expression and/or function of the nucleic acid by affecting, for example, transcription and/or stability (*e.g.,* of mRNA). Such elements can be included in the nucleic acid molecule to obtain optimal performance of the nucleic acid.

The nucleic acid for use in the present invention can be obtained from a variety sources using known methods. For example, the nucleic acid encoding a vacuolar pyrophosphatase (*e.g.,* AVP1) for use in the present invention can be derived from a natural source, such as tobacco, bacteria, tomato or corn. In one embodiment, the nucleic acid encodes a vacuolar pyrophosphatase that corresponds to a wild type of the transgenic plant. In another embodiment, the nucleic acid encodes a vacuolar pyrophosphatase that does not correspond to a wild type of the transgenic plant. Nucleic acid that alters (enhances, inhibits) expression of the endogenous vacuolar pyrophosphatase of the plant into which the exogenous nucleic acid is introduced (*e.g.,* regulatory sequence) can also be chemically synthesized, recombinantly produced and/or obtained from commercial sources.

A variety of methods for introducing the nucleic acid of the present invention into plants are known to those of skill in the art. For example, Agrobacterium-mediated plant transformation, particle bombardment, microparticle bombardment (*e.g.,*

U.S. Patent No. 4,945,050; U.S. Patent No. 5,100,792) protoplast transformation, gene transfer into pollen, injection into reproductive organs and injection into immature embryos can be used. The exogenous nucleic acid can be introduced into any suitable cell(s) of the plant, such a root cell(s), stem cell(s) and/or leaf cell(s) of the plant.

In one embodiment, a construct comprising a vacuolar pyrophosphatase gene operably linked to a promoter designed to overexpress the vacuolar pyrophosphatase (*e.g.,* an expression cassette) or a construct designed to downregulate endogenous pyrophosphatase is used to produce the transgenic plants of the present invention. As used herein the term "overexpression" refers to greater expression/activity than occurs in the absence of the construct. In a particular embodiment, a construct comprising an AVP1 gene operably linked to a chimeric promoter designed to overexpress the AVP1 or designed to downregulate endogenous pyrophosphatase is used to produce the transgenic plants of the present invention. More particularly, the present invention relates to a construct wherein the AVP1 gene is operably linked to a double tandem enhancer of a 35S promoter.

Any suitable plant can be used to produce the transgenic plants of the present invention. For example, tomato, corn, tobacco, rice, sorghum, cucumber, lettuce, turf grass, ornamental (*e.g.,* larger flowers, larger leaves) and legume plants can be transformed as described herein to produce the transgenic plants of the present invention. In addition, the transgenic plants of the present invention can be grown in any medium which supports plant growth such as soil or water (hydroponically).

The present invention also encompasses methods of making a transgenic plant which is tolerant to salt. In one embodiment, the method comprises introducing into one or more cells of a plant exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells in the plant, thereby producing a transgenic plant which is tolerant to salt. In another embodiment, the method comprises introducing into one or more cells of a plant a nucleic acid construct which is designed to overexpress AVP1 to yield transformed cells, thereby producing a transgenic plant which is tolerant to salt. The methods of making a transgenic plant can further comprise

regenerating plants from the transformed cells to yield transgenic plants and selecting a transgenic plant which is tolerant to salt. The transgenic plants produced by these methods are also encompassed by the present invention.

The transgenic plants of the present invention are useful for a variety of purposes. As described herein, the plant components involved in an intracellular cation detoxification system have been identified by complementing salt-sensitive mutants of the budding yeast *Saccharomyces cerevisiae*. The present invention relates to a method of bioremediating soil comprising growing one or more transgenic plants and/or progeny thereof in the soil, wherein the transgenic plants and/or progeny thereof comprise exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant. In another embodiment, the present invention relates to a method of removing cations (*e.g.*, monvalent and/or divalent cations) from a medium which can support plant growth (*e.g.*, soil, water) comprising growing one or more transgenic plants and/or progeny thereof in the medium, wherein the transgenic plants and/or progeny thereof comprise exogenous nucleic acid which alters expression of vacuolar pyrophosphatase in the plant. For example, the method can be used to remove sodium (Na), lead (Pb), manganese (Mn) and/or calcium (Ca) ions from a medium which supports plant growth.

Furthermore, it has been shown herein that the transgenic plants of the present invention are larger than the corresponding wild type plants (Example 3). Thus, the present invention provides for a method of increasing the yield of a plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby increasing the yield of the plant. The present invention also relates to a method of making a plant which is larger than its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant which is larger than its corresponding wild type plant. The method can further comprise regenerating plants from the transformed cells to yield transgenic plants and

selecting a transgenic plant which is larger than its corresponding wild type plant, thereby producing a transgenic plant which is larger than its corresponding wild type plant. Also encompassed by the present invention is a method of making a transgenic plant (*e.g.*, an ornamental plant) having increased flower size compared to its corresponding wild type plant comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant having increased flower size compared to its corresponding wild type plant.

The present invention also provides for a method of producing a transgenic plant which grows in salt water comprising introducing into one or more cells of a plant nucleic acid which alters expression of vacuolar pyrophosphatase in the plant to yield transformed cells, thereby producing a transgenic plant which grows in salt water. As used herein, "salt water" includes water characterized by the presence of salt, and preferably wherein the concentration of salt in the water is from about 0.2M to about 0.4M. In one embodiment, salt water refers to sea water.

The transgenic plants of the present invention can also be used to produce double transgenic plants which are tolerant to salt wherein a plant is transformed with exogenous nucleic acid which alters expression of a vacuolar phosphatase and exogenous nucleic acid which alters expression of another protein involved in sequestration of cations and/or detoxification in plants. In one embodiment, the present invention relates to a double transgenic plant which is tolerant to salt comprising one or more plant cells transformed with exogenous nucleic acid which alters expression of a vacuolar pyrophosphatase and an Na^+/H^+ antiporter in the plant. In one embodiment, the vacuolar pyrophosphatase is AVP1 or a homologue thereof and the Na^+/H^+ antiporter is AtNHX1 or a homologue thereof. The present invention further relates to a transgenic progeny of the double transgenic plant, as well as seeds produced by the transgenic plant and a progeny transgenic plant grown from the seed.

Investigation of the role of intracellular organelles in cation homeostasis via the identification and manipulation of key transporters is described herein. Most of these

intracellular organelles, including clathrin-coated vesicles, endosomes, Golgi membranes and vacuoles have acidic interiors (Xie, X. S., *et al., J. Biol. Chem., 264*:18870-18873 (1989)). This acidification is mediated by a proton-translocating electrogenic ATPase and in plant vacuoles also via a pyrophosphate-driven proton pump V-PPase (Davies, J.M., *et al.*, The Bioenergetics of Vacuolar H+ Pumps. In: *Leigh R.A., Sanders, D., (eds) The Plant Vacuole*, pp. 340-363, Academic Press, San Diego (1997); Zhen, R.G., *et al.*, "The Molecular and Biochemical Basis of Pyrophosphate-Energized Proton Translocation at the Vacuolar Membrane Academic Press Limited (1997)). There exists a requirement of anion transport to maintain net electroneutrality (al-Awqati, A., *Curr. Opin. Cell. Biol., 7*:504-508 (1995)). The yeast member of the CLC voltage-gated chloride channel superfamily, Gef1, is required for copper loading in late-Golgi vesicles and for cation sequestration in the prevacuolar compartment in yeast (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 95*:4046-4050 (1998); Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 96*:1480-1485 (1999); Example 1). Furthermore, it has been shown that the defects of *gef1* mutants can be suppressed by the introduction of the prototype member of the CLC superfamily, the *Torpedo marmorata CLC-0* or by the introduction of *Arabidobsis thaliana CLC-c* and *CLC-d* chloride channel genes (Hechenberger, M., *et al., J. Biol. Chem., 271*:33632-33638 (1996); Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 95*:4046-4050 (1998)). While not wishing to be bound by theory, two observations led to the proposal of a model for Na^+ sequestration in yeast described herein (Figures 3A and 3B). First, *gef1* mutants are sensitive to high NaCl concentrations. Second, the Na^+/H^+ exchanger Nhx1 localized to the prevacuolar compartment (Nass, R., *et al., J. Biol. Chem., 273*:21054-21060 (1998)). This model posits that Na^+ sequestration by Nhx1 depends on the vacuolar H^+-ATPase and Gef1, the chloride channel. Gef1-mediated anion influx allows the establishment by the vacuolar H^+-ATPase of a proton gradient sufficient in magnitude to drive the uphill accumulation of Na^+ via Na^+/H^+ exchange.

This model is entirely consistent with the physiological data on the role of the vacuole in cation detoxification in higher plants. As described in Example 1, to test this

sequestration model, mutant yeast strains (*enal*) lacking the plasma membrane sodium efflux pump, which therefore must rely on the internal detoxification system in order to grow on high salt, were constructed. In theory, increasing the influx of protons into the postulated endosomal compartment should improve Na^+ sequestration via the Nhx1 exchanger. In order to increase the H^+ availability the *A. thaliana* gain-of-function mutant gene *AVP1-D* that codes for the vacuolar pyrophosphate-energized proton pump was expressed (Figure 3B) (Zhen, R.G., *et al., J. Biol. Chem., 272*:22340-22348 (1997)). This plant pump expressed in yeast restored the Na^+ resistance of the test strain, but only if the strain had functional *NHX1* and *GEF1* genes. Furthermore, Gef1p and Nhx1p colocalize within a common organelle, the prevacuolar compartment (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 96*:1480-1485 (1999)). These results strongly support the model in Figures 3A and 3B and indicate that the yeast prevacuolar compartment can be used to identify the elusive plant transporters involved intracellular sodium detoxification.

Yeast and plant cells share pathways and signals for the trafficking of vesicles from the Golgi network to the vacuole (Neuhaus, J.M., *et al., Plant Mol. Biol., 38*:127-144 (1998); (Paris, N., *et al., Plant Physiol., 115*:29-39 (1997); Sato, M.H., *et al., J. Biol. Chem., 272*:24530-24535 (1997); Vitale, A.V., *et al., Trends Plant Sci., 4*:148-154 (1999)). As shown herein, intracellular Na^+ detoxification in yeast requires functional Na^+/H^+ exchanger (Nhx1) and chloride channel (Gef1), and they colocalize to a prevacuolar compartment (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 96*:1480-1485 (1999)). As described in Example 1, to further test the utility of this system, an *Arabidopsis thaliana* homologue of the yeast *NHX1* gene *(AtNHX1)* was cloned and its function in the *nhx 1* yeast mutant was tested. The *AtNHX1* gene was able to suppress partially the cation sensitivity phenotypes of *nhx1* mutants. Further support for the role of the *Arabidopsis AtNHX1* gene in salt homeostasis came from the observation that its expression is induced in salt-stressed plants (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 96*:1480-1485 (1999)). A recent report shows that the overexpression of *AtNHX1* gene in transgenic *Arabidopsis thaliana* promotes sustained growth in soil watered with

200 mM NaCl plys 1/8 M.S. salts under short-day cycle conditions (Apse, M., *et al., Science, 285*:1256-1258 (1999)). It is worth noting that every addition of 1/8 M.S. salts provides 2.5 mM potassium reducing the stringency of the NaCl stress, and that a short-day cycle reduces oxidative stress. As described in Example 2, transgenic plants that overexpress the *AtNHX1* were generated (35*SAtNHX1* transgenics).

In plants, most of the transport processes are energized by the primary translocation of protons. H^+-translocating pumps located at the plasma membrane and tonoplast translocated H^+ from the cytosol to extracellular and vacuolar compartments, respectively (Rea, P.A., *et al.,* Tonoplast Adenosine Triphosphate and inorganic Pyrophosphatase. In: *Methods Plant Biochem.*, pp. 385-405, Academic Press Limited, London (1990)). The plant tonoplast contains two H^+-translocating pumps; the V-ATPase and the inorganic pyrophosphatase or V-PPase. Their action results in luminial acidification and the establishment of a H^+ electrochemical potential gradient across the tonoplast (Davies, J.M., *et al.,* The Bioenergetics of Vacuolar H^+ Pumps. In: *Plant Vacuole*, pp. 340-363, Leigh, R.A., Sanders, D. (eds.), Academic Press, San Diego (1997)). The vacuolar membrane is implicated in a broad spectrum of physiological processes that include cytosolic pH stasis, compartmentation of regulatory Ca^{2+}, sequestration of toxic ions such as Na^+, turgor regulation, and nutrient storage and retrieval. The vacuole constitute 40 to 99% of the total intracellular volume of a mature plant cell. The vacuolar proton pumping pyrophosphatase is a universal and abundant component of plant tonoplast capable of generating a steady-state transtonoplast H^+ electrochemical potential similar or greater than the one generated by the V-ATPase (Rea, P.A., *et al.,* Tonoplast Adenosine Triphosphate and Inorganic Pyrophosphatase. In:*Methods Plant Biochem.*, pp. 385-405, Academic Press Limited, London (1990)). Pyrophosphate (PPi) is a by-product in the activation or polymerization steps of a wide range of biosynthetic pathways and in plants serves as an alternative energy donor to ATP for sucrose mobilization via sucrose synthase, for glycolysis via PPi: fructose-6-phosphate phosphotransferase and for tonoplast energisation via the vacuolar proton pumping pyrophosphatase (Stitt, M., *Bot. Acta 111*:167-175 (1998)).

As described in Example 1, the overexpression of the *A. Thaliana* gain-of-function mutant gene *AVP1-D* increases the intracellular detoxification capability in yeast (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 96*:1480-1485 (1999)). The rationale behind this approach is that an increased influx of H^+ into the vacuolar compartment should improve Na^+ sequestration via the Nhx1 exchanger. As described in Example 3, in order to test this hypothesis in plants, a transgenic *Arabidopsis thaliana* plant was engineered to overexpress the *AVP1* wild-type gene using the double tandem enhancer of the 35S promoter (Topfer, R., *et al., Nucl. Acid Res., 15*:5890 (1987)). *AVP1* encodes the pyrophosphate-energized vacuolar membrane proton pump from *Arabidopsis* (Zhen, R.G., *et al., J. Biol. Chem., 272*:22340-22348 (1997)). Previous investigations suggest that the *AVP1* gene is present in a single copy in the genome of Arabidopsis (Kim, Y., *et al., Plant Physiol., 106*:375-382 (1994)), however, a sequence homologous, but not identical, to *AVP1* on chromosome one has been tentatively designated as ORF F9K20.2 on BAC F9K20 by the Arabidopsis Genome Initiative (AGI).

Five different lines of 35*SAVP1* plants showed an enhanced salt tolerance as compared to wild-type plants in the T2 stage. However, the most dramatic phenotype was apparent in the homozygous T3 plants. These transgenic plants are larger than wild-type plants. Furthermore, homozygous 35*SAVP1* plants show sustained growth in the presence of 250 mM NaCl plus 1/8 M.S. salts when grown in a 24 hours light regimen. Interestingly, when 35*SAVP1* plants were grown under short-day cycle conditions sustained growth in the presence of 300 mM NaCl plus 1/8 M.S. salts was observed.

Hydroponic culture increases plant growth and provides stress-free root and shoot material (Gibeaut, D.M., *et al., Plant Physiol.*, 317-319 (1997)). Another important advantage of hydrophonic culture is that we can alter the ionic composition in a more accurate manner than in soil. These advantages could be important for the physiological studies of salt stress. As described in Example 4, wild type and 35*SAVP1* transgenic plants were grown hydroponically. Under such conditions the size

differences in root, leaves and stems among wild type and 35*SAVP1* transgenic plants are dramatic. To learn about the salt tolerance of these plants under hydroponic conditions, NaCl concentration were increased stepwise by 50 mM every 4 days (Apse, M., *et al., Science, 285*:1256-1258 (1999)). 35*SAVP1* transgenic plants appear healthy in the presence of 200 mM NaCl while wild type controls show severe deleterious effects in their leaves and stems.

Genetic engineering promises to transform modern agriculture. Salinization of soil due to irrigation has rendered much land unusable for crop production. Described herein is a strategy using genetic and molecular biological approaches to improve the intracellular Na^+ detoxification capabilities of crops. The fact that genetically engineered *Arabidopsis thaliana* plants that overexpress either *AVP1* (the pyrophosphate-energized vacuolar membrane proton pump, this work) or *AtNHX1* (the Na^+/H^+ antiporter, (Apse, M., *et al., Science, 285*:1256-1258 (1999)) and this work) are capable of growing in the presence of 200 mM NaCl strongly supports the strategy described herein. It is likely that a double transgenic plant will show a further enhanced salt-tolerant phenotype. Furthermore, it is expected that these *Arabidopsis thaliana* transporters or their counterparts will be able to perform similar function in important agricultural crops. The increased size of 35S *AVP1 Arabidopsis* transgenic plants also contribute to future food security, namely potential yield increases in genetically engineered crops.

EXEMPLIFICATION

Example 1 The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast

Materials and Methods

Yeast strains and Plasmids. All strains used are isogenic to W303 (*ura3-1 can1-100 leu2-3, 112trp1-1 his3-11*, (Gaxiola, R.A., *et al., EMBO J., 11*:3157-3164 (1992)). Plasmids pRG52 (Δ*gef1::HIS3*) (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 95*:4046-4050 (1998)) and pRG197 (Δ*nhx1::HIS3*) were used to construct the deletions

of *GEF1* and *NHX1* genes, yielding strains RGY85 and RGY296, respectively. The *enal::HIS3* mutant was obtained from Fink Lab collection (L5709). Transformation was performed by using the lithium acetate method (Gietz, D., *et al., Nucleic Acids Res., 20*:1425 (1992)). Double mutants RGY324 (*gef1::HIS3 enal::HIS3*), RGY326 (*nhx1::HIS3 enal::HIS3*), and RGY343 (*gef1::HIS3 nhx1::HIS3*) were obtained by crossing the single-mutant strains. Double mutants were identified among the meiotic progeny by scoring for the phenotypes associated with each of the single mutants. Sporulation, tetrad dissection, and mating types were scored as described (Guthrie C. and Fink, G.R., *Guide to Yeast Genetics and Molecular Biology* (Academic, San Diego (1991)). Cells were grown in YPD (1% yeast/2%peptone/2% dextrose; Difco), YPGAL (1% yeast/2%peptone/2% galactose; Difco), SD (Difco; Synthetic medium with 2% Dextrose), or APG (APG is a synthetic minimal medium containing 10 mM arginine, 8 mM phosphoric acid, 2% glucose, 2 mM $MgSO_4$, 1 mM KCl, 0.2 mM $CaCl_2$, and trace minerals and vitamins) (Rodriguez-Navarro, A. and Ramos, J., *J. Bacteriol., 159*:940-945 (1984)). $MnCl_2$ (Sigma), tetramethylammonium chloride (Sigma), NaCl (Sigma) , or hygromycin-B (Sigma were added as indicated.

Wild type, L5709 (*enal::HIS3*), RGY324 (*gef1::HIS3 enal::HIS3*), and RGY326 (*nhx1::HIS3 enal::HIS3*) strains were transformed with pYES2 vector (Invitrogen) and plasmid pYES2-*AVP1*-E229D described in ref. Zhen, R.G., et al., J. Biol. Chem., 272:22340-22348 (1997). The strain RGY343 (*gef1::HIS3 nhx1::HIS3*), used for histochemical analysis, was transformed with pRG151 (*GEF1-GFP*) (Gaxiola, R.A., *et al. Proc. Natl. Acad. Sci. USA, 95*:4046-4050 (1998)) and with pRIN73 [*NHX1-(HA)*$_3$] (Nass, R., and Rao, R., *J. Biol. Chem., 273*:21054-21060 (1998)).

Wild-type and RGY296 (*nhx1::HIS3*) strains were transformed with vector pAD4 (Ballester, R., *et al., Cell, 59*:681-686 (1989)). RGY296 (*nhx1::HIS3*) was transformed with pRG308 (*ADH1::AtNHX1*) (see *Cloning of AtNHX1*).

Determination of Intracellular Sodium and Potassium content. Cells were grown overnight in SD-ura medium (Difco; synthetic medium with 2% dextrose without uracil). YPGAL (1% yeast extract/2% peptone/2% galactose; Difco) media was

inoculated with the overnight stocks and grow to an A_{600} of 0.6. At this OD, NaCl was added to a final concentration of 0.7 M. The cells incubated for 6 h, harvested by centrifugation, washed two times with 1.1 M sorbitol and 20 mM $MgCl_2$, and entracted with water for 30 min at 95°C. The amount of Na^+ and K^+ in cells was determined at the University of Georgia Chemical Analysis Laboratory by an Inductively Coupled Plasma-MS (see http://www.rserv/uga.edu/rsnew/chemicalanalysis/). Intracellular cation concentrations were estimated as described (Gaxiola, R.A., *et al., EMBO J., 11*:3157-3164 (1992)) by using the intracellular water value calculated for cells grown in 1M NaCl.

Immunofluorescence. The strain RGY343 (*gef1::HIS3 nhx1::HIS3*) was grown in SD-ura, -leu medium (Difco; synthetic medium with 2% dextrose without uracil and leucin) to mid-logarithmic phase, 0.1 mg/ml hygromycin B was added, and the culture was incubated for 1 h at 30°C. Cells were fixed with 3.7% formaldehyde (Sigma) for 45 min at room temperature without agitation. Spheroplast formation, permeablization, washing, and antibody incubation was performed as described (Pringle, J., *et al.*, in *Immunofluorescence Methods for Yeast*, eds. Guthrie, C. And Fink, G.F. (Academic, Sand Diego), Vol. 194 pp.565-602 (1991)). MAB HA11 used as first antibody was from Babco (Richmond, CA). Cy3-conjugated goat antimouse IgG was from Jackson Immunoresearch. 4',6-Diamidino-2-phenylindole (Sigma) was added to mounting medium to stain mitochondrial and nuclear DNA.

Subcellular Fractionation and Western Analysis. The strain RGY343 (*gef1::HIS3 nhx1::HIS3*) was grown in APG medium (pH 7.0), and lysates fractioned on a 10-step sucrose density gradient as described (Nass, R. and Rao, R., *J. Biol. Chem., 273*:21054-21060 (1998)). Aliquots of individual fractions (100 µg) were subjected to SDS/PAGE and transferred to nitrocellulose as described (Nass, R. and Rao, R., *J. Biol. Chem., 273*:21054-21060 (1998)). Western blots were probed with monoclonal anti-GFP (green fluorescent protein) antibody (1:10,000 dilution; CLONTECH), anti-hemagglutinin antibody (1:10,000 dilution: Boehringer Mannheim), and peroxidase-

coupled goat anti-mouse antibody (1:5,000;) and developed by using the ECL enhanced chemiluminescence system (Amersham Pharmacia).

Plant Strains, Growth conditions and RNA Preparation. *A. thaliana* plants (ecotype Columbia) were grown aseptically on unsupplemented plant nutrient agar without sucrose (Haughn, G.W. and Somerville, C., *Mol. Gen. Genet., 204*:430-434 (1986)) for 15 days at 19°C and under continuous illumination. NaCl or KCl was added to a final concentration of 250 mM, and the plants were incubated for 6 h. Total RNA from tissue of salt-treated and untreated plants was isolated (Niyogi, K.K. and Fink, G.R., *Plant Cell, 4*:721-733 (1992)), Hybond-N (Amersham) membranes were hybridized with a ^{32}P-Labeled DNA probe from plasmid pRG308. Hybridization was performed at 65°C overnight. Washes were performed at 65°C with 0.2% standard saline citrate (SSC)/0.1% SDS (Ausebel, F., *et al.*, *Curr. Protocols in Mol. Biol.* (Wiley, NY) (1988)). *18S* probe was used as loading control (Unfried, I., *et al., Nucleic Acids Res., 17*:7513 (1989)). MACBAS 2.4 program was used to quantify the relative amount of RNA.

Cloning of *AtNHX1*. *AtNHX1* was cloned from a phage cDNA library of *A. thaliana* (Kieber, J.J., *et al., Cell, 72*:427-441 (1993)) (obtained from the Arabidopsis Biological Resource Center) by probing with an expressed sequence tag (Arabidopsis Biological Resources Center, DNA Stock Center) containing a partial clone. A full-length clone (2.1 kB) was ligated into vector pSK2 (Stratagene) at the *Not*I sit, generating plasmid pRG293. The *AtNHX1* ORF was amplified via PCR by using pRG293 as template and GGCCCGGG*ATGGATTCTCTAGTGTCGAAACTGCCTTCG* (SEQ ID NO: 5) (italicized bases correspond to nucleotides 1-30 of the ORF) and T7 oligonucleotides. The PCR product was then digested with *Xba*I and *Sal*I and ligated into pAD4 vector generating plasmid pRG308. The *AtNHX1* ORF was sequenced to verify the fidelity of the PCR product. The full-length sequence is longer than the ORF reported by the Arabidopsis Genome Initiative (A TM021B04.4), and has been deposited in GenBank (accession no. AF106324).

Results

The *Arabidopsis* Vacuolar H^+ -Pyrophosphatase (Avp1) Confers Salt Tolerance to Yeast *ena1* Mutants. To determine the components of the intracellular system required for sodium detoxification, an *ena1* mutant that lacks the plasma membrane sodium efflux pump and therefore must rely on the internal detoxification system to overcome sodium toxicity was used. Growth of the *ena1* strain is sensitive to low concentrations of sodium (200 mM), concentrations that do not inhibit the growth of wild-type strains. The sequestration model (Nass, R. and Rao, R., *J. Biol. Chem., 273*:21054-21060 (1998) and, Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 95*:4046-4050 (1998)) predicts that the *ena1* strain would become salt tolerant if one could enhance the availability of protons in the postulated endosomal compartment. With increased influx of protons, cytoplasmic Na^+ would be sequestered via the Nhx1 exchanger. The yeast vacuolar ATPase is a multisubunit protein, so it is difficult to increase its activity by overexpressing any one of its subunits. However, it is possible to increase the influx of protons by expressing the *A. thaliana AVP1* gene in yeast. This gene encodes a single polypeptide that, when expressed in yeast, is capable of pumping protons into the lumen of the vacuole (Kim, E.J., *et al., Proc. Natl. Acad. Sci. USA, 91*:6128-6132 (1994)). To ensure maximum activity of this proton pump, the E229D gain-of-function mutant of the *AVP1* gene (*AVP1-D*) that has enhanced H^+ pumping capability was expressed (Zhen, R.G., *et al., J. Biol. Chem., 272*:22340-22348 (1997)).

Overexpression of *AVP1-D* restored salt tolerance to salt-sensitive *ena1* mutants. The restoration of salt tolerance to an *ena1* strain by *AVP1-D* requires functional *NHX1* and *GEF1* genes: *ena1nhx1 AVP1-D* and *ena1 gef1 AVP1-D* strains are salt sensitive.

Expression of *Arabidopsis* vacuolar pyrophosphatase *AVP1* in *ena1* mutants: Vector pYES2 (Invitrogen) was introduced into wild-type, *ena1, ena1 nhx1*, and *ena1 gef1* mutants. Plasmid pYes2-*AVP1-D* (Zhen, R.G., *et al., J. Biol. Chem., 272*:22340-22348 (1997)) was introduced into *ena1, ena1 nhx1,* and *ena1 gef1* mutants. Five-fold serial dilutions (starting at 10^5 cells) of each strain were plated on YPGAL (1% yeast extract/2% peptone/2% galactose) with or without 0.5 M NaCl and incubated at 30°C

for 2 days. Figures 1A and 1B show intracellular concentrations of Na^+ and K^+. Exponentially growing cells (wild-type and *ena1* transformed with pYES2 vector and *ena1, ena1 nhx1,* and *ena1 gef1* mutants carrying pYes2-*AVP1-D*) were exposed to 0.7M NaCl or 6 hours. Total cell extracts were prepared (see Materials and Methods), and Na^+ and K^+ concentrations were determined. There is a consistent reduction in total cell Na^+ in the *ena1 AVP-D* strain. The reason for this reduction is unknown.

The intracellular Na^+ and K^+ contents of wild-type strains and of strains carrying various mutations affecting sodium tolerance were determined after 6 h of exposure to media supplemented with 0.7 M NaCl (Figures 1A and 1*B*). The intracellular Na^+ content in the *ena1* mutant is 8-fold higher than in the wild-type strain. The *ena1 AVP-D* strain is salt-resistant, even though its intracellular Na^+ content is 4-fold higher than that of the wild type. In *enal AVP1-D* strains lacking either *gef1* or *nhx1* (i.e., *ena1 gef1* or *ena1 nhx1*), the Na^+ content is not reduced to the extent that it is in *GEF1 NHX1* strain. Taken together, the genetic and physiological data are consistent with the model that Nhx1, Gef1 and Avp1 cooperate to sequester sodium internally.

The intracellular K^+ content correlates with salt tolerance and is inversely correlated with the Na^+ content of our strains (Figure 1*B*). The wild-type K^+ concentration is ≈ 100 mM but is reduced to 20 mM in the *ena1* mutant. Interestingly, in an *ena1* strain that overexpresses the *AVP1-D* gene, the intracellular concentration of K^+ is restored almost to wild-type levels (Figure 1B). However, *AVP1-D* overexpression fails to restore wild-type levels of intracellular potassium unless both NHx1 and GEF1 are functional (see the double mutants *ena1 nhx1* or *ena1 gef1* in Figure 1*B*).

The *NHX1* and *GEF1* genes, which have been identified as important in sodium detoxification, are also required for the detoxification of other cations. Growth of *gef1* and *nhx1* mutants in the presence of toxic cations: Five-fold serial dilutions (starting at 10^5 cells) of the indicated strains were grown at 30°C for 2 days on YPD (1% yeast extract/2% peptone/2% dextrose) with the addition of either 3 mM $MnCl_2$, 0.45 M tetramethylammonium (TMA), or 0.05 mg/ml hygromycin B (HYG) as indicated.

For example, *gef1* mutants are sensitive to 3 mM $MnCl_2$, 0.45 M tetramethylammonium chloride and to 0.05 μg/ml hygromycin-B. The *nhx1* mutant is also sensitive to tetramethylammonium chloride and hygromycin. The extreme sensitivity of the *nhx1* mutant to hygromycin provides an important tool for assaying nhx1 function.

Gef1p and Nhx1p Colocalize. The sequestration model postulates not only a functional connection between the anion channel Gef1 and sodium exchanger Nhx1 but also predicts that these two proteins colocalize within a common compartment. Because previous studies indicated that Nhx1 localizes to a prevacuolar compartment (Nass, R. and Rao, R., *J. Biol. Chem., 273*:21054-21060 (1998)), two types of experiments were performed to determine whether Gef1 and Nhx1 proteins colocalize to this compartment.

Distribution of fluorescence and immunodetection of subcellular fractions in *gef1 nhx1* cells transformed with two constructs: a *GEF1-GFP* fusion and a *NHX1-(HA)*$_3$-tagged fusion were determined. The strain RGY419 (*gef1 nhx1*) was transformed with plasmids pRG151; GEF1-GFP and pRIN73; NHX1-$(HA)_3$. Transformants were grown in SD (Difco; synthetic medium with 2% dextrose). When the cells reached OD_{600} = 0.5, hygromycin B (Sigma) was added to a final concentration of 0.1 mg/ml and the cells were incubated for 40 min at 30°C. Cells were fixed and stained with antibodies to HA epitope and 4',6-diamidino-2-phenylindole (DAPI). Cells were viewed by charge-coupled device microscopy and optically sectioned by using a deconvolution algorithm (Scanalytics, Billerica, MA) (Kennedy, B.K., *et al., Cell, 89*:381-391 (1997)); (Bar = 1 μm.).

It was found that hemagglutinin (HA)-tagged Nhx1 and Gef1-GFP fusion protein colocalize as shown via epifluorescence deconvolution microscopy (Fig. 3*A*). Persistence of signal coincidence on 90° rotation of the image further supports colocalization of the two transporter proteins in these cells.

The colocalization of Nhx1 $(HA)_3$ and GEF1-GFP is also supported by the comigration of the two proteins in sucrose density gradients of membrane preparations

obtained from cells expressing the tagged proteins. The strain RGY419 (*gef1 nhx1*) transformed with plasmids pRG151; GEF1-GFP and pRIN73; NHX1-$(HA)_3$ was grown in APG medium (Rodriguez-Navarro, A. and Rea, P.A., J. Biol. Chem., 159:940-945 (1984)), converted to spheroplasts, lysed, and fractionated on a 10-step sucrose gradient (18-54%) as described (Sorin, A., *et al., J. Biol. Chem., 272*:9895-9901 (1997) and Antebi, A. and Fink, G.R., *Mol. Biol. Cell, 3*:633-654 (1992)). Western blots showed the distribution of Gef1-GFP and Nhx1-HA (see Example 1, Materials and Methods).

The sedimentation behavior of the membrane fraction containing both proteins is consistent with that of a prevacuolar compartment (Nass, R. and Rao, R., *J. Biol. Chem., 273*:21054-21060 (1998)). Gef1-GFP (but not Nhx1) is also present in Golgi fractions, consistent with previous studies (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 95*:4046-4050 (1998), Schwappach, B., *et al., J. Biol. Chem., 273*:15110-15118 (1998)).

An *A. thaliana* Homologue of NHX1 Functions in Yeast. The yeast strain described herein provides an important tool for identifying genes that mediate salt tolerance in other organisms. To test the utility of this system, a sequence from *Arabidopsis* (See *Materials and Methods*) with very high homology to the *S. cerevisiae NHX1* ORF was identified and used an expressed sequence tag (see *Materials and Methods*) to obtain a full-length clone of this *Arabidopsis* gene. An alignment of the amino acid sequences of Nhx1 homologues from *Arabidopsis* (AtNhx1), human (HsNhe6), and yeast (ScNhx1) reveals segments of amino acid identity and similarity within predicted transmembrane domains (Fig. 2). However, it is important to note that despite these relationships, neither the – nor the C-terminal regions of AtNhx1 and ScNhx1 show a high degree of homology (Fig. 2). A characteristic of mammalian Na^+/H^+ antiporters is their inhibition by amiloride. A putative amiloride binding site (^{163}DVFFLFLLPPI^{173}) (SEQ ID NO: 4) has been defined via point mutants in the human NHE1 antiporter gene (Counillon, L., *et al., Proc. Natl. Acad. Sci. USA, 90*:4508-4512 (1993)). AtNhx1, HsNhe-6 and ScNhx1 have an almost identical sequence (Fig. 2). However, our attempts to inhibit the activity of either Nhx1 or AtNhx1 in yeast cultures with amiloride were unsuccessful.

The extreme sensitivity of yeast *nhx1* mutants to hygromycin permitted the testing of whether the cloned *Arabidopsis AtNHX1* ORF could provide Na^+/H^+ exchange function in yeast. Vector pAD4 (Ballester, R., *et al., Cell, 59:*681-686 (1989) was introduced into wild-type and *nhx1* strains. Plasmid pRG308; ADH; *At*NHX1 was introduced into *nhx1* mutants as indicated. Five-fold serial dilutions (starting at 10^5 cells) of the indicated strains were grown at 30°C for 2 days on YPD (-) or on YPD supplemented with 0.05 mg/ml hygromycin (+). Serial dilutions of the same strains were grown on APG medium (see Materials and Methods) (-) or on APG supplemented with 0.4 M NaCl (Rodriguez-Navarro, A. and Ramos, J., *J. Bacteriol., 159:*940-945 (1984).

The *At NHX1* gene is capable of suppressing the hygromycin sensitivity of the *nhx1* mutant. The *AtNHX1* gene also suppressed the NaCl sensitivity of *nhx1* mutant but only under conditions in which the K^+ availability was reduced. However, *AtNHX1* was not capable of rescuing the Na^+ - sensitive growth phenotype of the double mutant *ena1 nhx1* overexpressing the *AVP1-D* gene.

Further support for the role of the *Arabidopsis AtNHX1* gene in salt homeostasis came from an analysis of its expression in salt-stressed plants. Plants were grown for 15 days under standard conditions and then exposed for 6 h to either 250 mM NaCl or KCl. The NaCl stress increased *AtNHX1* mRNA levels 4.2-fold, whereas KCl promoted only a 2.8-fold increase. This increase in mRNA level produced by sodium resembles that described for the yeast *NHX1* gene (Nass, R. and Rao, R., *J. Biol. Chem., 273:*21054-21060 (1998)). RNA tissue blot hybridized with *At*NHX1. Ten micrograms of total RNA from 15-day old plants exposed to 250 mM NaCl or Kcl for 6 h and a control grown without salt was subjected to electrophoresis on a denaturing formaldehyde gel. The blot was hybridized with a probe internal to *At*NHX1 ORF. An *18S* ribosomal probe was used as a loading control.

Discussion

The studies described herein provide evidence for the importance of the prevacuolar pH for intracellular Na^+ sequestration in yeast. Overexpression of the plant H^+ -pyrophosphatase (Avp1) confers salt tolerance to yeast only in those strains containing a functional chloride channel (Gef1) and the Na^+/H^+ exchanger (Nhx1).

These data support a model in which the Nhx1 Na^+/H^+ exchanger acts in concert with the vacuolar ATPase and the GEF1 anion channel to sequester cations in a prevacuolar compartment. Several studies suggest that the prevacuolar compartment may be derived both from the plasma membrane and the late Golgi. These vesicles are likely involved in the assembly of the vacuole or delivery of cargo to this organelle. It is reasonable to expect that these prevacuolar vesicles detoxify cations by sequestration, thereby lowering their concentrations in the cytoplasm and in other organelles.

The yeast system described herein permits the functional assessment of diverse heterologous proteins in salt tolerance: chloride channels, H^+ pumps, and Na^+/H^+ exchangers and other cation/H^+ exchnagers or cation/bicarbonate symporters. The system is robust and flexible. The function of the *Arabidopsis* chloride channels (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 95*:4046-4050 (1998), Hechenberger, M., *et al., J. Biol. Chem., 271*:33632-33638 (1996)), H^+ pump, and Na^+/H^+ exchanger can be assayed in the corresponding yeast mutant. Despite the inability of *At NHX1* to suppress all the phenotypes of the yeast *nhx1* mutant, the fact that it suppresses some phenotypes, coupled with the DNA homology between *AtNHX1* and yeast *NHX1*, indicates that the plant gene carries out a similar function to that of the yeast homologue. The observation that the *AtNHX1* gene suppresses the sensitivity of the *nhx1* mutant to hygromycin but provides only a weak Na^+ detoxification phenotype could be a consequence either of differential regulation of the transporters in the two organisms or of distinct cation transport selectivities.

The regulation of *AtNHX1* by salt and the ability of the plant gene to suppress the yeast *nhx1* mutant suggest that the mechanism by which cations are detoxified in yeast and plants may be similar. Indeed, previous work suggested that vacuolar sodium

accumulation in salt-tolerant plants may be mediated by a tonoplast Na^+/H^+ antiporter that utilizes the proton-motive force generated by the vacuolar H^+-ATPase (V-ATPase) and/or H^+ -translocating pyrophosphatase (V-PPase; refs. Barkla, B.J., *et al., Symp. Soc. Exp. Biol., 48*:141-153 (1994), Zhen, R.G., *et al., The Molecular and Biochemical Basis of Pyrophosphate-Energized Proton Translocation at the Vacuolar Membrane* (Academic, San Diego), Kirsh, M, *et al., Plant Mol. Biol., 32*:543-547 (1996)).

The finding described herein that both *gef1* and *nhx1* mutants are hypersensitive to hygromycin indicate that the level of resistance to hygromycin depends on the function of the vacuolar and prevacuolar organelles. Yeast mutants impaired in K^+ uptake (*trk1*) are hypersensitive to hygromycin (Madrid, R., *et al., J. Biol. Chem., 273*:14838-14844 (1998)); reduced K^+ uptake hyperpolarizes the plasma membrane potential and drives the uptake of alkali cations such as hygromycin. Mutations that reduce the H^+ pumping activity of the plasma membrane H^+ -ATPase, Pmal, depolarize the plasma membrane potential and confer resistance to hygromycin (McCusker, J.H., *et al., Mol. Cell. Biol., 7*:4082-4088 (1987)). Thus, mutants such as *gef1* or *nhx1* that affect the pH or membrane potential of the vacuolar and prevacuolar compartments may be expected to affect hygromycin compartmentation.

Example 2 Transgenic plants that overexpress the *AtNHX1*

Transgenic plants that overexpress the *AtNHX1* were generated using Agrobacterium-mediated plant transformation. The transgenic *AtNHX1* was expressed using a double tandem enhancer of the 35S promoter of CaMV (Topfer, R., *et al., Nucl. Acid Res., 15*:5890 (1987)). T3 transgenic plants are less affected than wild type controls when watered with 300 mM NaCl.

15 wild-type plants and 15 *35SAtNHX1* transgenic were grown on a 12 hours-day cycle for 20 days. During this period plants were watered every 5 days with a diluted nutrient solution (1/8 M.S. salts). 200 mM NaCl was added to the watering solution at day 21 and at day 33 plants were watered with a nutrient solution containing 300 mM NaCl. Plants were photographed 10 days after the last NaCl treatment.

Example 3 Salt-stressed wild type plants and 35*SAVP1* transgenics

Transgenic plants that overexpress AVP1 were generated using Agrobacterium-mediated plant transformation. The transgenic *AVP1* was expressed using a double tandem enhancer of the 35S promoter of CaMV (Topfer, R., *et al., Nucl. Acid Res., 15*:5890 (1987)). 15 wild-type plants and 15 35*SAVP1* transgenics were grown on a 24 hours-day cycle for 16 days. During this period plants were watered every 4 days with a diluted nutrient solution (1/8 M.S. salts). 200 mM NaCl was added to the watering solution at day 17 and at day 27 plants were watered with nutrient solution containing 250 mM NaCl. Plants were photographed 10 days after the last NaCl treatment. Identical conditions and treatment as described in Example 2 were used.

These transgenic plants are larger than wild-type plants. Furthermore, homozygous 35*SAVP1* plants show sustained growth in the presence of 250 mM NaCl plus 1/8 M.S. salts when grown in a 24 hours light regimen. Interestingly, when 35*SAVP1* plants were grown under short-day cycle conditions (12 hour day/light cycle) sustained growth in the presence of 300 mM NaCl plus 1/8 M.S. salts was observed.

Example 4 Hydroponically grown wild type and 35*SAVP1* transgenic plants

Hydroponically grown wild type and 35*SAVP1* transgenic plants were generated. 65 days old wild type and 35*SAVP1* transgenic plants grown in solution culture on a 12 hour light cycle.

Wild type and 35*SAVP1* transgenic plants were also grown in solution culture on a 12 hours light cycle for 20 days. Starting at day 21, NaCl concentration was increased in a stepwise fashion by 50 mM increments every 4 days. Plants were photographed after 4 days in the presence of 200 mM NaCl.

Example 5 Double Transgenic Plant with *35S AVP1* and *35S AtNHX1*

Overexpression of the pyrophosphate-energized vacuolar membrane proton pump AVP1 likely increases the availability of H^+ in the lumen of the vacuole, and the AtNHX1 Na^+/H^+ antiporter uses these H^+ to sequester Na^+ cations into the vacuole.

Therefore, higher expression of these transporters likely maximizes the sequestration capability of the vacuole. To generate transgenic *Arabidopsis* plants that overexpress both genes *AVP1* and *AtNHX1*, T3 *35S AVP1* plants are used as females and T3 *35S AtNHX1* plants are used as males. Female plants are hand-emasculated and anthers from freshly opened flowers of donor plants are harvested. With these anthers the emasculated plants are pollinated by touching the anthers onto the stigmas. The pollinated flowers are labeled and any remaining opened or unopened flowers from the same female plant are removed to avoid any confusion at harvest. The harvested seeds are sterilized using a 50% sodium hypochloride solution and mixed vigorously for 5 minutes and rinsed with water thoroughly. The sterilized seeds are stored in soft agar over night at 4°C. Then they are sprinkled onto solidified kanamycin-hygromycin selective medium. The *35S AVP1* construct has the neomycin phosphotransferase II gene that confers kanamycin tolerance in plants while the *35S AtNHX1* construct has a modified hygromycin B phosphotransferase that confers hygromycin tolerance in plants. The resistant seedlings are transplanted into soil and to the hydroponic media to be tested for their salt-tolerant phenotype. A transgenic *Arabidopsis thaliana* plant to overexpress the *A. thaliana* gain-of-function mutant gene *AVP1-D* (Zhen, *et al., J. Biol. Chem., 272*:22340-22348 (1997)) is engineered using the same double tandem enhancer of the 35A promoter described above (Topfer, R., *et al., Nucl. Acid Res., 15*:5890 (1997)). Plants overexpressing the gain of function mutant gene will likely show an enhanced phenotype. These plants are characterized in parallel with the *35SAVP1*, *35S AtNHX* singles and doubles trangenics. The *A. thaliana* gain-of-function mutant gene *AVP1-D* is subcloned into plasmid pRT103 carrying the 35S promoter and the polyadenylation signal of CaMV (Topfer, R., *et al., Nucl. Acid Res., 15*:5890 (1997)). A HindIII fragment containing the chimeric *35SAVP-D* gene is subcloned into pBIBhyg (Becker, D., *Nucl. Acid Res., 18*:203 (1990)). The resulting T-DNA vector is transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation, and used for subsequent vacuum infiltration of *Arabidopsis thaliana* ecotype Columbia (Bechtold, N., *et al., C.R. Jeances Acad. Sci. Ser. III Sci. Vie, 316*:1194-1199 (1993)).

Integration is confirmed on Southern blots of T3 plants and expression monitored on Northern blots of positive T3 plants.

Example 6 Comparative Transport Study With Vacuoles From The Roots Of Wild-Type and *35S AVP1* Transgenic Plants

The purpose of this study is to determine if the vacuoles of *35S AVP1* transgenic plants show a higher proton transport activity dependent on pyrophosphate. These determinations are done with root and shoot tissues separately from plants grown hydroponically. The transgene could show a tissue-specific regulation despite the 35S promoter.

In order to compare the PPI-dependent H^+ translocation activities of wild-type and *35S AVP1* transgenic plants sealed tonoplast-enriched vesicles from roots and leaves of the above plants are prepared. The homogenization and differential centrifugation procedure described by Rea and Turner (Rea, P.A., *et al.,* Tonoplast Adenosine Triphosphate and Inorganic Pyrophosphatase. In: *Meth. Plant Biochem.*, pp. 385-405, Academic Press limited, London (1990)) is followed. H^+ translocation is assayed fluorimetrically using acridine orange (2.5 μM) as transmembrane pH difference indicator in assay media containing vacuole membrane-enriched vesicles as described by Rea and coworkers (Zhen, R.G., *et al., J. Biol. Chem., 272*:22340-22348 (1997)). The assay media contains 300μM Tris-PPi, 50mK KC1, 2.5 μM acridine orange, 5mM Tris-Mes (pH 8.0). Intravesicular acidification is triggered with the addition of 1.3 mM MgSO4 and terminated with the addition of the protonophore FCCP at 2.5μM. Fluorescence is measured at excitation emission wavelengths of 495 and 540 nM, respectively, at a slit width of 5 nM (Zhen, R.G., *et al., J. Biol. Chem., 269*:23342-23350 (1994)). A further test to support that the H^+ translocation is *AVP1* driven is the addition of the specific inhibitor aminomethylenediphosphonate (Zhen, R.G., *et al., Plant Physiol., 104*:153-159 (1994)).

Example 7 Determination Of The Na^+/K^+ Ratios In Leaves And Stems Of The Transgenic Plants

These measurements indicate to whether or not the transgenic plants described herein have an increased vacuolar capacity to sequester Na^+ in their leaves cells or elsewhere. Toxic concentrations of NaC1 build up first in the fully expanded leaves where NaC1 is compartmentalized in the vacuoles. Exposure to NaC1 can disrupt or reduce K^+ uptake leading to K^+ deficiency and growth inhibition (Wu, S.J., *et al., Plant Cell, 8:*617-627 (1996). A cytosolic consequence of reduced K^+ content and high Na^+ is the inhibition of important enzymes. An example of such enzymes is the 3'(2'), 5'-bisphosphate nucleotidase of yeast whose activity is more sensitive to Na^+ when K^+ content is low (Murguia, *J.R., et al., Science, 267:*232-234 (1995). To determine the Na^+/K^+ ratios in leaves and stemS wild-type and *35S AVP1 /35S AtNHX1* double and single transgenics in hydroponic conditions (Gibeaut, D.M., *et al., Plant Physiol.,* 317-319 (1997) are grown. NaCl is added to the growth media in a stepwise fashion starting with 50 mM up to 250mM (Apse, M., *et al., Science, 285:*1256-1258 (199). At every point the rosette and the stems of the treated plants are collected and their weight is determined. The samples are dried out in an oven at 80°C and their dry weight is determined. The dry samples are boiled in a determined volume of water and their Na^+ and K^+ contents determined via atomic absorption spectrophotometry (Apse, M., *et al., Science, 285:*1256-1258 (1999); Gaxiola, R., *et al., Embo J., 11:*3157-2164 (1992)).

Example 8 Determination Of Whether *35S AVP1* Transgenic Plants Are Larger Because Their Cells Are Larger Or Because They Have More Cells, Or Both

The shoot meristems labeling index is compared with one of the wild-type plants. Morphological and anatomical observations measuring and counting cells of leaves, roots and stems are performed. To determine if *35S AVP1* transgenic plants are larger because they have more cells, their shoot meristems labeling index is compared with the one of wild-type plants. To measure the DNA synthesis or cell proliferation 5-

Bromo-2'-deoxy-uridine (BrdU) that can be incorporated into DNA in place of thymidine is used. Cells that have incorporated BrdU into DNA are detected using a monoclonal antibody against BrdU monoclonal antibody and an anti-mouse Ig-alkaline phosphatase as a second antibody. The bound anti-BrdU monoclonal antibody is visualized by light microscopy and the ratio between DAPI stained and BrdU positives established. The protocol is a modification of the one published by Chiatante and coworkers (Levi, M., *et al., Physiol. Plant. 71*:68-72 (1987)) and the BrdU labeling and detection kit II from Boehringer Mannheim. The plants are exposed for different times to the BrdU labeling medium and then fixation, paraffin embedding and sectioning is performed as described by Meyerowtz and coworkers (Drews, G., *et al., Plant Mol. Biol. Rep., 5*:242-250 (1988)). For observation of leaf tissue, fresh tissues are embedded in 5% agarose and slice them with a microslicer. For primary root observation, seedlings are fixed for 4hr in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde at room temperature, dehydrate them in graded ethanol series, permeate them with xylene, and infiltrate them with paraffin. Eight-micrometer sections are stained with 0.05% toluidine blue and cells are counted under a microscope. As an alternative for the visualization and determination of cell size the method described by Greenberg and coworkers (Rate, *et al., The Plant Cell, 11*:1695-1708 (1999)) is followed.

Example 9 Isolation Of Mutants In The Transporters

Genetic approaches are very powerful in analyzing complex biological traits (Serrano, R., *Crit. Rev. Plant Sci., 13*:121-138 (1994)) Reverse genetics is a very important new tool for plant biologists. The generation of a good collection of tagged knockouts by Sussman and coworkers (Krysan, P., *et al., Proc. Natl. Acad. Sci. USA, 93*:8145-8150 (1996)) has open a very important avenue for the analysis of gene disruptions in *Arabidopsis.* the Arabidopsis Knock-out Facility of the University of Wisconsin Madison (http://www.biotech.wisc.edu/NewServicesAndResearch/Arabidopsis) is used to search

among the 60,480 Arabidopsis (ecotype WS) lines that have been transformed with the T-DNA vector pD991 for the presence of T-DNA inserts within *At*CLC-*c*, *At*CLC-*d*, AVP1, *At*NHX1 and their homologues. The phenotypes of the above knock-outs will shed light towards the understanding of the physiological roles of these transporters in normal and stress conditions. An initial characterization of the knockout plants includes testing for their salt tolerance and their Na^+/K^+ ratios. The generation of double knock-outs via crosses help to further understand the interaction among the transporters as well as the crosses with the *35S AVP1* and the *35S AtNHX1* transgenic plants.

To search for *Arabidopsis* knock-out PCR primers are designed following the guidelines detailed in the University of Wisconsin web site. Tested primers are sent to UW-Madison, where 62 PCR reactions that are sent to us for Southern blot analysis are performed. Positive PCR products are sequenced. If the sequence reveals that there is a T-DNA inserted within the gene the gene specific primers are sent for another set of PCR reactions in order to determine which of the 9 possible pools of 225 contains the knockout. After identifying the pool of interest, 25 tubes of seeds are screened for the individual plant carrying the T-DNA knock-out.

Example 10 Cation Detoxification In Plant Cells

The studies described herein together with other evidence strongly indicate that yeast and plants share pathways and signals for the trafficking of vesicles from Golgi network to the vacuole (Gaxiola, R., *et al., Proc. Natl. Acad. Sci. USA*, 96:1480-1485 (1999); Marty, F., "The Biogenesis of Vacuoles: Insights from Microscopy. In: *The Plant Vacuole*, 1-42, Leigh, R.A. and Sanders, D., Academic Press, San Diego (1997); Bassham, D.C., *et al., Plant Physiol, 117*:407-415 (1998)). Without wishing to be bound by theory, it is likely that in both systems a prevacuolar compartment is a dynamic entity that detoxifies the cytoplasm from toxic cations and delivers its cargo either to the vacuole, or directly to the cell exterior. Both the Gefl chloride channel and Nhx1 Na^+/H^+ exchanger have been localized to the yeast prevacuolar compartment (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 96*:1480-1485 (1999)). The behavior

of the Gef1-GFP chimera in yeast cells in vivo have been monitored indicating that its localization varies depending the environmental conditions. Furthermore, it has been shown that two of the four *A. thaliana* CLC chloride channel genes CLC-*c* and -*d* are capable of suppressing *gef1* mutant phenotypes implying a similar localization (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 95*:4046-4050 (1998)). In order to understand how and where this cation detoxification takes place in plant cells the intracellular localization of GFP chimeras of AVP1, AtNHX1 and AtCLC-c and -d (Hong, B., *et al., Plant Physiol., 119*:1165-1175 (1999)) is monitored *in vivo*. Confocal microscopy is also used to address colocalization of the different transporters. For this purpose HA-tagged versions or antibodies of the transporters under study are required (Guiltinan, M.J., *et al., Meth. Cell Biol., 49*:143-151 (1995); Jauh, G. -Y., *et al., Plant Cell, 11*:1867-1882 (1999); Mullen, R.T., *et al.., Plant. J., 12*:313-322 (1997)).

For the constructions of the GFP-chimeras the soluble versions GFP with improved fluorescence in A. thaliana reported by Davis and Viestra (Davies, S.J., Viestra, R.D., "Soluble derivatives of green fluorescent protein (GFP) for use in *Arabidopsis thaliana*, http:/ / brindabella.mrc-lmb.cam.ac.uk/IndexGFP.html (1998)) are used. Two types of GFP-chimeras are made, namely a set under the regulation of the native promoter and another set under the regulation of the 35S promoter. The resulting T-DNA vectors containing the GFP-chimeras are transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation, and used for subsequent vacuum infiltration of *Arabidopsis thaliana* ecotype Columbia (Bechtold, N., *et al., C.R. Jeances Acad. Sci. Ser. III Sci. Vie, 316*:1194-1199 (1993)). For the hemagglutinin (HA) epitope tagging a PCR strategy designed for yeast but modified to tag plant genes expressed in yeast vectors is used. Futcher and coworkers designed vectors containing the URA3 yeast gene flanked by direct repeats of epitope tags (HA) (Schneider, B.L., *et al., Yeast, 11*:1265-1274 (1995)). Via PCR the tag-URA3-tag cassette is amplified such that the resulting PCR fragment possess homology at each end to the gene of interest. *In vivo* recombination in yeast is then used to direct the integration of the PCR-chimera to the plasmid carrying the plant ORF of interest, transformants are selected by the

URA$^+$ phenotype. The URA3 gene is "popped out" when positive transformants are grown in the presence of 5-fluoro-orotic acid. The vector carrying the plant gene has a selection marker different than the URA3 gene.

Example 11 Further applications of the yeast model

Gain of function mutants of the AtNHX that enhance salt tolerance of transgenic plants are generated using the yeast system. This is accomplished by mutagenizing the cloned gene to make a mutant library. This library is used to transform the salt sensitive yeast mutant *enal* and clones with an enhanced salt tolerant phenotype will be identified and retested. The other genes that show similarity to the AtNHX1 gene reported by the Arabidopsis Genome Initiative (AGI) are expressed in yeast. It is likely that some of these AtNHX1 homologues are plasma membrane transporters, so their function in yeast should be pH dependent. Thus the precise composition and pH of the medium used for screening is crucial for success. Identification of plasma membrane transporters helps to engineer plants with an enhanced salt tolerance due to a reduced sodium uptake. In addition, plant cDNA expression libraries in yeast are used to identify other families of transporters involved in NaCl detoxification.

To generate gain of function mutants of the AtNHX a method for introducing random mutations developed by Stratgene (Epicurian Coli XL1-Red competent Cells Cat#200129) is used. The method involves the propagation of a cloned gene into a strain deficient in the three primary DNA repair pathways. The random mutation rate in this strain is about 5000-fold higher than that of wild-type. A library of the mutated AtNHX gene is transformed into the *enal* yeast mutant and screened for salt tolerance. Yeast transformation is performed as described by Schiestl and coworkers (Gietz, D., *et al., Nucl. Acid Res. 20*:1425 (1992)). An alternative to the XL1-Red random mutagenesis strategy is a PCR approach described by Fink and coworkers (Madhani, H.D., *et al., Cell, 91*:673-684 (1997)). To test AtNHX1 homologues the same strains and conditions used for AtNHX1 (Gaxiola, R.A., *et al., Proc. Natl. Acad. Sci. USA, 96*:1480-1485 (1999)) are used initially. However, if these screening strains and/or

conditions do not work new ones are worked out. It is likely that when dealing with plasma-membrane AtNHX1 homologues pH conditions of the assay media are crucial.

Example 12 Hydroponic Culture of Transgenic Plants

The reduced availability of fresh water for standard agriculture may force the use of alternative agricultural arts. It is conceivable that with salt tolerant crops the use of hydroponics with seawater will create a new era in crop production. As described herein, conditions for hydroponics culture of *Arabidopsis* plants have been established and their performance in increasing concentrations of NaCl in their media have been tested. Transgenic plants are challenged with a commercial seawater formula that contains the complete ionic composition present in the oceans.

35SAVP1, 35SAtNHX1 single and double transgenics are grown together with wildtype *Arabidopsis thaliana* plants under hydroponic conditions for four weeks in a short day illumination cycle (Gibeaut, D.M., *et al., Plant Physiol.*, 317-319 (1997)). Then every four days an equivalent to 50mM NaCl of Tropic Marin sea salt (www.thatpetplace.com) is added. This artificial sea water mix includes all of the other major and trace elements present in real sea water. Growth is monitored and physiological parameters, such as sodium content and distribution is determined as described in previous sections.

The effects of the overexpression of these *Arabidopsis thaliana* proton transporters (AVP1 and AtNHX1) in more agriculturally important plants such as tomato are examined. The tomato homologues of AVP1 and AtNHX1 are isolated and the corresponding chimeras to overexpress them are constructed (Bidone, S., *et al., Eur. J. Biochem., 253:* 20-26 (1998); Burbidge, A., *et al., J. Exper. Botany, 48*:2111-2112 (1997)). The genes are introduced via *Agrobacterium*-mediated infection of calli. Tissue culture methods are used to regenerate transformed plants. The plants are assayed for salt tolerance as well as physiological parameters, such as sodium content and distribution. Increasing the salt-tolerance of tomato plants will likely have important economic repercussions. A positive result indicates that the sequestration

model described herein is also applicable to an important crop. Tomato transformation with 35S AVP1 and with 35S AtNHX1 constructs is performed as described by McCormick (McCormick, S., Transformation of tomato with *Agrobacterium tumefaciens*. In: *Plant Tissue Culture Manual*, pp. 1-9, Lindsey, K. (ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands (1991)). T0 and T1 transgenics are analyzed by polymerase chain reaction and DNA gel blotting for the presence and copy number of AVP1 and AtNHX1 transgenes. Heterozygous and homozygous plants are identified after segregation analysis of each transcend within T1 seeds. Homozygous plants are assayed for salt tolerance and as well as physiological parameters, such as sodium content and distribution. Degenerated oligos based on conserved sequences present in AVP1 and AtNHX1 homologues are designed. These degenerated primers are used in RT-PCR reactions with cDNAs made from poly(A)+RNA from tomato. The resulting PCR fragments are used as probes to isolate the full length cDNA clones from commercial libraries (i.e. Stratagene Cat#936004). A similar strategy was described by Caboche and coworkers (Quesada, A., *et al., Plant Mol. Biol., 34*:265-274 (1997)).

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.